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Research Article

Highly sensitive detection of invasive shore crab (*Carcinus maenas* and *Carcinus aestuarii*) larvae in mixed plankton samples using polymerase chain reaction and restriction fragment length polymorphisms (PCR-RFLP)

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Abstract

The brachyuran crab genus *Carcinus* consists of two species, *C. maenas* and *C. aestuarii*, both of which have invaded multiple regions of the globe. *C. maenas* has proven a particularly adept invader, establishing introduced populations on every non-polar continent outside its native range. This species has also exhibited the capacity to spread rapidly once established and has potential for significant ecological and economic impacts throughout its introduced range. The possibility of both species invading additional coastal ecosystems, and the importance of larval dispersal—both current-driven and ballast water-mediated—to the successful establishment and expansion of introduced populations, recommend the development of rapid and cost-effective tools for detecting and monitoring *Carcinus* larvae in environmental samples. We have developed a PCR-RFLP approach that enables the specific and highly sensitive detection of both *C. maenas* and *C. aestuarii* in mixed plankton samples, including those drawn from ballast water. Our approach successfully identifies specimens from throughout the native and introduced ranges of both species, and excludes all non-target brachyuran species tested, including a number of species whose ranges overlap with those of the Carcinus species. Sensitivity of our PCR-RFLP assay is extremely high, allowing the detection of single stage I zoea in over 1 gram (filtered weight) of mixed non-target plankton. The assay also successfully detected single larvae in mixed plankton derived from ballast water, indicating the potential utility of this approach as a tool for targeted screening of *Carcinus* sp. in ship's ballast.

Key words: invasive species, marine invasions, monitoring, DNA identification

Introduction

The European green crab *Carcinus maenas* (Linnaeus, 1758) is a notoriously successful invasive species, with established non-native populations in Australia, South Africa, Japan, Atlantic and Pacific North America, and, most recently, Argentina (Hidalgo et al 2005; Carlton and Cohen 2003). In some of these regions the species has expanded rapidly from the site of initial introduction; on the Pacific coast of North America, a population introduced to San Francisco Bay in the late 1980s had expanded over approximately 1700 kilometers of coastline by the year 2000 (Yamada 2000; Yamada and Hunt 2000). Both ballast water-mediated translocation (Carlton and Cohen 2003; Cohen et al 1995) and natural current-driven dispersal of larval crabs (Thresher et al 2003; Yamada and Hunt 2000) have been implicated in the spread of *C. maenas* at both global and regional scales, and are likely to be the primary contemporary vectors of introduction for the species. A number of physiological and life history characteristics contribute to the species' global success: *C. maenas* exhibits extremely high fecundity (Broekhuysen 1936; Cohen et al 1995), possesses long-lived feeding larval stages (Queiroga et al 2002; Dawirs 1985; Cohen et al 1995), is tolerant of wide temperature and salinity ranges (Broekhuysen 1936), and is omnivorous and opportunistic in its feeding habits (Cohen et al 1995).

In addition to its capacity to colonize novel habitats and expand rapidly on a regional scale, *C. maenas* has the potential to cause significant

ecological and economic disturbance in recipient ecosystems. The species has been shown to be capable of considerably altering physical habitat (Davis et al 1998; Lafferty and Kuris 1996) and reducing abundance of native benthic micro- and macrofauna (Grosholz and Ruiz 1995; Grosholz et al 2000; Lafferty and Kuris 1996). Of particular concern is the implication of *C. maenas* in the failure of commercial clam fisheries in Atlantic North America (Lafferty and Kuris 1996; Glude 1955; Floyd and Williams 2004), and the potential for similar negative effects in other regions where the species is introduced (Walton et al 2002). In addition, *C. maenas* is likely to directly compete with a number of native crustaceans, including commercially important species such as Dungeness crab (*Cancer magister* Dana, 1852) on the Pacific coast of North America (McDonald et al 2001; Jamieson et al 1998; Grosholz et al 2000; Grosholz and Ruiz 1995).

While *C. maenas* has achieved deserved notoriety, its congener *Carcinus aestuarii* (Nardo, 1847) has exhibited less extensive success as an invasive species. A native of Mediterranean Europe and North Africa, *C. aestuarii* has been introduced to both Japan and South Africa (Carlton and Cohen 2003; Geller et al 1997). Morphological and genetic data suggest that *C. aestuarii*, rather than *C. maenas*, is the dominant introduced crab species in Japan's Tokyo and Dokai Bays (Geller et al 1997; Yamada and Hauck 2001), although the possibility remains that these populations may actually be of hybrid origin (Bagley and Geller 2000; Yamada and Hauck 2001). Little is known about the potential impacts of this species in its introduced range, but studies have shown it to practice the same opportunistic omnivory as its sister species (Chen et al 2004).

Here we describe the development and evaluation of a PCR-based assay for the detection of *C. maenas* and *C. aestuarii* larvae in environmental samples. We employ a two-step process for species-specific identification, involving PCR amplification using genus-specific primers followed by species-specific restriction enzyme digestion of the resulting amplicon. We show that this approach is capable of correctly identifying *C. maenas* and *C. aestuarii* specimens from throughout both the native and introduced ranges of the two species. In addition, we demonstrate specificity of the assay by testing genus-specific PCR on non-target crab species, including a number of species whose ranges overlap with those of the *Carcinus* species. The sensitivity of the assay regularly enables detection of single *C. maenas* larvae in mixed plankton samples (including ballast water samples) even when background non-target biomass is extremely high. This specific and highly sensitive assay for detection of *Carcinus* larvae should provide a valuable tool for managers and researchers interested in assessing the dispersal of these species.

Materials and methods

Sample collection and processing

Brachyuran crab tissue samples utilized in this study were obtained from a number of sources. Recently collected, preserved (95% ethanol) or frozen specimens of crab species commonly found on the Pacific coast of North America were provided by Sylvia Yamada (Oregon State University) and Greg Jensen (University of Washington). Additional preserved (70% ethanol) tissue samples were obtained from Rob Toonen (University of Hawaii). Non-*Carcinus* crab samples were processed for whole genomic DNA using the DNeasy Tissue Kit (Qiagen) on either gill or leg muscle tissue. All crab DNA samples were normalized to approximately 5 ng/µl. First stage zoea of *Carcinus maenas*, preserved in 95% ethanol, were provided by Uwe Nettlemann and Klaus Anger (Alfred Wegener Institute).

Development of Carcinus-specific PCR-RFLP assay

Partial sequences of the mitochondrial cytochrome C oxidase subunit I (COI) gene were either provided by Joseph Roman (University of Vermont), generated at the EPA Molecular Ecology Research Branch in Cincinnati, or obtained from Genbank (all non-*Carcinus* haplotypes). In total, 99 *Carcinus* haplotypes (82 *C. maenas* and 17 *C. aestuarii*) and 34 haplotypes from non-*Carcinus* brachyuran crab species were aligned using ClustalX (Thompson et al 1997) and scanned by eye for conserved regions within the genus *Carcinus* and within the species *C. maenas*. Three regions were identified, two which were well conserved within the entire genus and one which was conserved only within *C. maenas* (Table 1). The former were chosen as sites for the design of *Carcinus*-specific primers CF3 (5'-TTAGGAGGGCCAG

Table 1. Alignment of *Carcinus* and non-*Carcinus* COI sequences at forward and reverse primer binding sites and internal *EcoNI* restriction site. All sequences are given from 5' to 3' in the direction of the COI open reading frame. Identities are indicated with a period (.), gaps with a dash (-). For *Carcinus* species, we indicate the number of individual haplotypes with the given COI sequence at forward, reverse, and internal regions. Non-*Carcinus* species marked with an asterisk (*) were included in specificity tests.

Species	Forward primer TTAGGAGGGCCAGATATAGCTTT	EcoNI site CCTTTAGCAGG	Reverse primer TATTATTATCGTTGCCGGTTTTAG	# of haplotypes
Carcinus maenas	.....	.....	.....	64
	.....	.....	.....T.....	2
	.....A.....	.....	.....	9
	.....	.....	.....A.....	1
	.....G.....	.....	.....	3
	.....	.....	..C.....	1
	.....	.....	.....C.....	1
	.....	.....	.....A.....	1
Carcinus aestuarii	.....	..AC....C..	.....A.....T.....	3
	.....	..GC....T..	.....A.....T.....	3
	.....	..GC....T..	.....AC....T.....	1
	.....	..AC....C..	.....A..A..T.....	1
	.....	...C....T..	.....A.....T.....	1
	.....A.....	..CC....C..	.....A.....T..C....	1
	.....A.....	..CC....C..	.....A..A..T..C....	6
	.....A.....	...C....C..	.....A..A..T..C....	1
Liocarcinus depurator	.....CT..T..C.....C..	..CC..T.G.C	.T...C....C..A..T..C....	
Necora puber	C....T.CT.....G.....	..C...T.T.C	.GC..C....TC.A..A.....	
Callinectes sapidus	C......CT..T.....C..	..CC.T..T.C	..C.TC.T..TC.A..T..A....	
Callinectes arcuatus	C......CT..T.....	.....C.C	.TC.CC.T..AC....T...C...	
Callinectes bellicosus	C......CT..T.....	.....G.C	.TC.TC.C..TC.C..T..CC...	
Portunus trituberculatus	.....CT..T.....C..	...C.TT.T.C	.TC.T.....TC.C..T...C.G.	
*Cancer gracilis**	.....CT..T.....	.....	..C.CC.C..TC.C..T...C...	
*Cancer productus**	.....CA..T.....C..	.....	.GC.GC....CC.C..T.....	
*Cancer japonicus**	C......CC..T.....G..A..	.....	.....C..A..T.....	
Cancer pagarus	.....CT..T.....	..CC....G..	..C.T.....TC.C..T.....	
*Cancer novazealandae**	C.G....C...T..C.....C..	.....	..C.TC....AC.C..T..C....	
*Hemigrapsus nudus**	C....G.C......C.....A..	..CC.CT.C.C	..C.T.....TC.T..A...C...	
*Hemigrapsus oregoniensis**	C......C.....G..	..C...T.T.C	..C.TC....TC.C..T.....	
Pachygrapsus crassipes	.....CT..T.....G..C..	.....G....C	.GC.T..G..C..A..C.....	
Chionoecetes bairdi	.....CC..T.....	..A.....C	.TC..C....TC....T..------	
Chionoecetes japonicus	.....CC..T.....	C	.TC..C....TC.A..T..------	
Chionoecetes opilio	C.G....CC..T.....C..	C	.TC.....TC.A..T..------	
Deckenia imitatrix	C......CT..T.....	..A.....T.C	.TC.TC.T..TC.N..A..------	
Erimacrus isenbeckii	.....G.C...T.....G.....	.....C.C	.CC.T.....TC.T..T..------	
Eriocheir formosa	.....T.CC..T.....A..	...C.G....C	.TC.TC....TC.T..C..------	
Eriocheir hepuensis	C......CC.....	C	.TC.....CC.T..A..------	
Eriocheir japonica	C......CC.....	C	.TC.T..G..TC.T..A..------	
Eriocheir leptognathus	.....CC..T.....	C	.T.....A..A..T..------	
Eriocheir rectus	.....T.CC..T.....A..	...C.G....C	.TC.TC....TC.T..C..------	
*Eriocheir sinensis**	C....G.CC.....	C	.TC.C.....CC.T..A..------	
Gaetice depressus	.....CA.....C.....	...C.T..T.C	..C.T.....TC.C..T..------	
Grapsus albolineatus	C......CT.....A..	...C......C	.CC.C.....TC.T..A..------	
Hemigrapsus sanguineus	C.C....CC.....C..	..AC.T....C	.GC.T.....T..A..A..------	
Hydrothelphusa madagascariensi	.....CT..T.....C..	..C.....C.C	.CC.TC.T..T.....T..------	
Liberonautes latidactylus	.....CT..T.....A..	.....A.	..C.TC.T..AC.C..C..------	
Plagusia immaculata	.....CT..T.....A..	.....C.C	.TC.GC.T..C..A..T..------	
Portunus trituberculatus	.....CT..T.....C..	...C.TT.T.C	.TC.T.....TC.C..T..------	
Potamon fluviatilis	.....CC.....A..	..A.....CTC	..C.CC.C..TC.T..A..------	
Potamonautes lividus	.....CC..T.....	.....T.C	.CC.TC.C..CC.A..T..------	
Ranina ranina	C.T....CC..T.....	.....T.C	.TC.T.....CC.T..T..------	
Rhithropanopeus harrisii	.....CC..T.....A..	.....T.C	..C.T.....T..A..T..------	
Sudanonautes africanus	.....CT..C.....A..	.....T.C	..C.C-....T-.A..T.------	
Sudanonautes faradjensis	.....CA..T.....A..	.....T.C	C.C.CC.T.TT-.A...N.-----	
*Telmessus cheiragonus**	.....T.CT..T.....C..	.....G..T.C	.TC.T.....TC.T..T..------	
Varuna litterata	.....CC..G..C.....	..AC....C.C	.C...C.T..T..A..A..------	

ATATAGCTTT-3') and CR3 (5'-CTAAAACCG GCAACGATAATAATAA-3'). Given the large amount of information available on this genus and significant variability within the COI locus, it was impossible to identify primer sites that were 100% conserved across all known haplotypes. As a result, a number of known *Carcinus* haplotypes exhibit mismatches within the primer binding sites (see Results). Primer sites were chosen so as to minimize these mismatches, particularly at the primer 3' end, and all mismatched haplotypes were tested directly for *Carcinus*-specific amplification. For the reverse primer site, only a subset (n = 14) of non-target COI sequences could be aligned over the entire primer binding site.

An internal *EcoNI* site was found to be 100% conserved within *C. maenas* (all haplotypes) but absent from all *C. aestuarii* haplotypes. This site was chosen for a species-specific diagnostic test based on restriction digestion of the *Carcinus*-specific amplicon. The predicted size of the *Carcinus*-specific PCR product is 348 basepairs for both *C. maenas* and *C. aestuarii*; digestion of this fragment by *EcoNI* is predicted to result in two products of 212 and 136 basepairs in *C. maenas*, but in an undigested 348 basepair product in *C. aestuarii*. We also identified two FokI restriction sites that flank the *EcoNI* site in *C. maenas*; species-specific RFLP identification was possible with this enzyme as well, and gave results equivalent to those obtained with *EcoNI* digestion (data not shown).

Molecular protocols

All PCR reactions were conducted in 15 µl total volume, and contained 0.5 units Taq DNA polymerase, 1x Mg-free PCR buffer, 1.7 mM MgCl2, 0.67 mM dNTPs, 1 µM each forward and reverse PCR primers, and 1 µL template DNA normalized to 5 ng/µL. For reactions designed to control for successful DNA extraction, universal COI primers COIF-PR115 (5'-TCWACNAAYC AYAARGAYATTGG-3') and COIR-PR114 (5'-ACYTCNGGRTGNCCRAARARYCA-3') were used (Folmer et al 1994), yielding an amplicon of approximately 700 bp from all crab species tested. Control PCR cycling parameters consisted of a 5 min denaturation cycle at 94º C, followed by 35 cycles of 1 min at 94º C, 1 min at 50º C, and 1 min at 72º C , with a final extension step of 15 min at 72º C. For *Carcinus*-specific amplification using primers CF3 and CR3, cycling parameters were identical except annealing was conducted at 65º C instead of 50º C.

All PCR products were run on 1.5% agarose gels (unless otherwise noted) and stained with ethidium bromide; gel images were digitized with KodakGL100 software. For specificity tests, equal volumes of both universal and *Carcinus*-specific PCR reactions (3 µL each) were run together in a single well to provide internal controls for each sample. All sensitivity tests were conducted with *C. maenas* larvae.

For *C. maenas*-specific RFLP detection, 5 µL of PCR product from *Carcinus*-specific PCR reactions was digested for 3 hours at 37º C in a 10 µL reaction including 1x buffer (NEB 4) and 1.5 units *EcoNI* (New England Biolabs). The entire volume of restriction digested product was loaded into a single well for visualization.

Preparation of plankton samples

Ballast water samples were collected from the vessel *General Villa* ported in Sacramento, CA (originating from Long Beach, CA) on August 28, 2004. Samples were collected by light trap (20 minute deployment) and preserved in 95% ethanol. Preserved plankton was filtered through 8 micron filters (Millipore) under vacuum, washed with 95% ethanol, and allowed to dry under vacuum for 3 minutes. For all sensitivity tests, between 1 and 3 mL settled plankton volume was filtered. Weights were recorded for all filtered and vacuum-dried samples before processing for DNA; these measurements are hereafter referred to as "filtered weight." For ballast plankton, experimental samples were spiked with individual *C. maenas* larvae (one larva per sample) after filtration.

To further explore the sensitivity of our approach, and due to limitations on the amount of plankton available from ballast samples, we generated larger scale mock plankton communities consisting of mixed, cultured zooplankton and containing up to 1.359 grams of biomass (filtered weight). These samples included *Artemia salina* (Linnaeus, 1758) nauplii, *Daphnia pulex* (Leydig, 1860), *Daphnia magna* (Straus, 1820), *Ceriodaphnia dubia* (Richard, 1894), and *Hyalella azteca* (Saussure, 1858) in unknown proportions. Live plankton were collected from culture and preserved in 95% ethanol. Approximately 5 mL settled plankton volume was used for each experimental sample; samples were spiked with 1, 5, 10, or 20

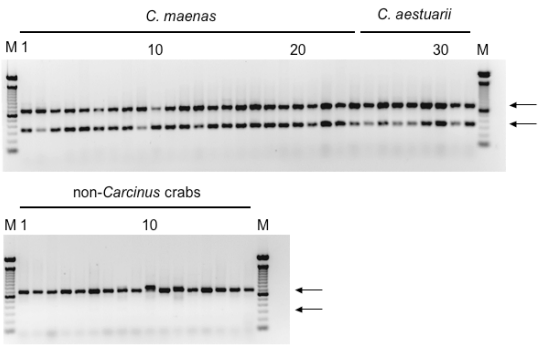

Figure 1. Specificity of *Carcinus*-specific PCR. Top panel: *C. maenas* and *C. aestuarii* DNA. Bottom panel: non-*Carcinus* crab DNA. Numbering is as in Table 2. High molecular weight band (~700 bp, top arrow) represents universal COI control; lower molecular weight band (348 bp, bottom arrow) represents *Carcinus*-specific COI product. M, 100 base pair ladder.

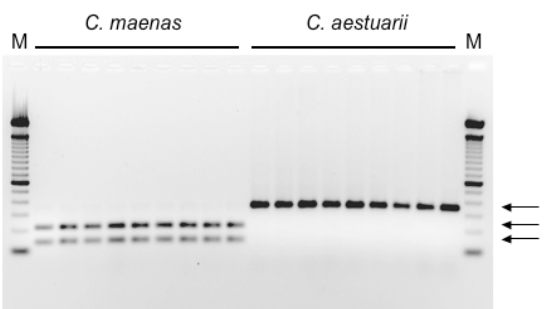

Figure 2. RFLP identification of *C. maenas* and *C. aestuarii*. *EcoNI* digests of *Carcinus*-specific products shown in Figure 1. Products were run on a 2% agarose gel to increase resolution for smaller fragments. Topmost arrow indicates undigested 348 bp *Carcinus*-specific PCR amplicon; lower two arrows indicate 212 and 136 bp *C. maenas*-specific digestion products. M, 100 base pair ladder. Samples correspond to *C. maenas* samples 1 through 9 and *C. aestuarii* samples 23 to 31 in Figure 1 and Table 2.

Carcinus maenas larvae and filtered as described above. To more closely mimic real-world attempts at detection, these samples were spiked and mixed thoroughly prior to filtration. This allows for the possibility of target organisms being lost in the filtration process. Filtered weights of were recorded for each sample before processing for DNA.

DNA extractions

For sensitivity tests, we processed filtered ballast water plankton, with or without added *C. maenas* larvae, using either the DNeasy Plant Kit (Qiagen) or the PowerSoil Kit (MoBio). These kits proved more efficient than the DNeasy kit for processing samples with more than 100 mg of filtered biomass. However, since mock plankton communities consisting of cultured zooplankton (with or without *C. maenas* larvae) contained far more biomass than the recommended limit for these kits, we utilized the PowerMax Soil Kit (MoBio) for processing these samples. All DNA extractions from plankton samples were conducted on dried, filtered plankton according to protocols provided by the commercial suppliers.

Results

Assay specificity

Amplification products from universal COI PCR demonstrate the presence of amplifiable mitochondrial DNA in all samples (Figure 1). *Carcinus*-specific PCR primers CF3 and CR3 amplified the predicted 348 basepair fragment from all tested *Carcinus* samples, but failed to amplify from all non-*Carcinus* samples at the 65º C annealing temperature (Figure 1 and Table 2). Sequence alignments suggest that our *Carcinus*-specific primers are unlikely to successfully amplify from any of the 34 non-*Carcinus* species investigated (Table 1); this prediction is confirmed for 8 species which were tested directly in specificity assays and whose COI haplotypes were included in the primer design (Figure 1). An additional 9 species were also shown to be excluded non-targets, despite not being included in the primer design stage of assay development. *Carcinus* samples include specimens from throughout both the native and introduced ranges of both *C. maenas* and *C. aestuarii* (Table 2). *Carcinus*-specific amplification was also successful at 65ºC for rare haplotypes that showed nucleotide mismatches within the conserved priming sites (Figure 1 and Table 2); amplification was successful in all such cases attempted, though not all data is shown here.

Carcinus-specific amplification products shown in Figure 1 were subjected to *EcoNI* digestion. Digestion of *C. maenas* products resulted in generation of the predicted 212 and 136 basepair fragments, whereas all *C. aestuarii* products remained undigested after 3 hours at 37º C (Figure 2 and Table 2).

Table 2. Samples used in specificity analysis. For non-*Carcinus* samples 5-17 and for all *Carcinus* samples, location indicates actual collection location. For non-*Carcinus* samples 1-4, location indicates only known native range. Specificity tests were either positive (+), negative (-), or not done (ND). Asterisks (*) indicate haplotypes with one or more mismatches in *Carcinus*-specific primer binding sites.

Sample#	Species	location	haplotype	Results of specificity tests		
				COI control	*Carcinus*-specific	*EcoNI* digest
1	*Carcinus maenas*	Barnstable, MA	1	+	+	+
2	*Carcinus maenas*	Gotegorg, Sweden	3*	+	+	+
3	*Carcinus maenas*	Murphy's Cove, Nova Scotia	5	+	+	+
4	*Carcinus maenas*	Murphy's Cove, Nova Scotia	6	+	+	+
5	*Carcinus maenas*	Mongstadt, Sweden	10	+	+	+
6	*Carcinus maenas*	Torshavn, Faroe Islands	13*	+	+	+
7	*Carcinus maenas*	Fowey, England	17	+	+	+
8	*Carcinus maenas*	Hoek van Holland, Netherlands	22	+	+	+
9	*Carcinus maenas*	Bremerhaven, Germany	23*	+	+	+
10	*Carcinus maenas*	Mongstadt, Sweden	25	+	+	+
11	*Carcinus maenas*	Trondheim, Norway	27	+	+	+
12	*Carcinus maenas*	Torshavn, Faroe Islands	29	+	+	+
13	*Carcinus maenas*	Bilbao	30	+	+	+
14	*Carcinus maenas*	Bilbao, Spain	32*	+	+	+
15	*Carcinus maenas*	Bilbao, Spain	33	+	+	+
16	*Carcinus maenas*	Bremerhaven, Germany	36	+	+	+
17	*Carcinus maenas*	Bremerhaven, Germany	37	+	+	+
18	*Carcinus maenas*	Fowey, England	43	+	+	+
19	*Carcinus maenas*	Oslo, Norway	49*	+	+	+
20	*Carcinus maenas*	Fowey, England	55	+	+	+
21	*Carcinus maenas*	Betanzos, Spain	90*	+	+	+
22	*Carcinus maenas*	Den Helder, Netherlands	93*	+	+	+
23	*Carcinus aestuarii*	Naples, Italy	58*	+	+	-
24	*Carcinus aestuarii*	Naples, Italy	59*	+	+	-
25	*Carcinus aestuarii*	Naples, Italy	60*	+	+	-
26	*Carcinus aestuarii*	Naples, Italy	61*	+	+	-
27	*Carcinus aestuarii*	Naples, Italy	62*	+	+	-
28	*Carcinus aestuarii*	Naples, Italy	63*	+	+	-
29	*Carcinus aestuarii*	Naples, Italy	64*	+	+	-
30	*Carcinus aestuarii*	Banyuls-sur-mer, France	73*	+	+	-
31	*Carcinus aestuarii*	Banyuls-sur-mer, France	76*	+	+	-
32	*Carcinus aestuarii*	Banyuls-sur-mer, France	100*	+	+	-
1	*Cancer novaezealandiae*	Australia/New Zealand		+	-	ND
2	*Cancer branneri*	Pacific coast, USA		+	-	ND
3	*Cancer japonicus*	Western Pacific		+	-	ND
4	*Cancer gracilis*	North Atlantic		+	-	ND
5	*Cancer borealis*	Pacific coast, USA		+	-	ND
6	*Pugettia producta*	Pacific coast, USA		+	-	ND
7	*Lophopanopeus bellus*	Pacific coast, USA		+	-	ND
8	*Hemigrapsus oregonensis*	Pacific coast, USA		+	-	ND
9	*Telmessus cheiragonus*	Pacific coast, USA		+	-	ND
10	*Cancer magister*	Pacific coast, USA		+	-	ND
11	*Hemigrapsus nudus*	Pacific coast, USA		+	-	ND
12	*Pugettia gracilis*	Pacific coast, USA		+	-	ND
13	*Scyra acutifrons*	Pacific coast, USA		+	-	ND
14	*Cancer productus*	Pacific coast, USA		+	-	ND
15	*Oregonia gracilis*	Pacific coast, USA		+	-	ND
16	*Pinnixa littoralis*	Pacific coast, USA		+	-	ND
17	*Eriocheir sinensis*	Pacific coast, USA		+	-	ND

Table 3. Description of sensitivity tests. Sample IDs are as shown in figure 3. Plankton biomass was measured as filtered weight. NA, not applicable.

Sample ID	Extraction method	Amount of plankton (mg)	Type of plankton	Number of larvae	Results of PCR screening	
					COI control	*Carcinus*-specific
A1	PowerSoil (mini)	0	NA	0	-	-
A2	PowerSoil (mini)	111	filtered ballast	0	+	-
A3	PowerSoil (mini)	145	filtered ballast	0	+	-
A4	PowerSoil (mini)	154	filtered ballast	1	+	+
A5	PowerSoil (mini)	175	filtered ballast	1	+	+
A6	PowerSoil (mini)	169	filtered ballast	1	+	+
A7	PowerSoil (mini)	156	filtered ballast	1	+	+
A8	PowerSoil (mini)	148	filtered ballast	1	+	+
B1	DNeasy Plant (mini)	0	NA	0	-	-
B2	DNeasy Plant (mini)	176	filtered ballast	0	+	-
B3	DNeasy Plant (mini)	121	filtered ballast	0	+	-
B4	DNeasy Plant (mini)	129	filtered ballast	1	+	+
B5	DNeasy Plant (mini)	178	filtered ballast	1	+	+
B6	DNeasy Plant (mini)	174	filtered ballast	1	+	+
B7	DNeasy Plant (mini)	154	filtered ballast	1	+	+
C1	PowerMax Soil (maxi)	0	NA	0	-	-
C2	PowerMax Soil (maxi)	745	combined culture	0	+	-
C3	PowerMax Soil (maxi)	1184	combined culture	1	+	+
C4	PowerMax Soil (maxi)	1359	combined culture	5	+	+
C5	PowerMax Soil (maxi)	966	combined culture	10	+	+
C6	PowerMax Soil (maxi)	868	combined culture	20	+	+

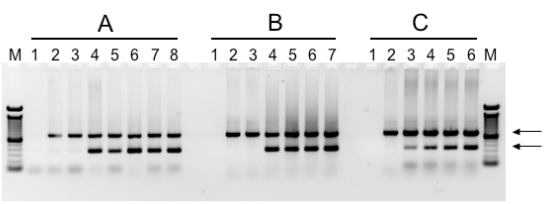

Figure 3. Sensitivity of *Carcinus*-specific PCR. Universal COI control PCR (high molecular weight band, top arrow) and *Carcinus*-specific PCR (low molecular weight band, bottom arrow) run for each sample. A, PowerSoil mini extractions; B, DNeasy Plant Kit mini extractions; C, PowerMax Soil Kit maxi extractions; M, 100 base pair ladder. Sample numbers correspond to Table 3. Unloaded lanes are shown between experiments for clarity of presentation.

Assay sensitivity

Carcinus-specific PCR was capable of consistently detecting single *C. maenas* larvae in spiked ballast water samples containing up to 178 milligrams of total non-target biomass (Figure 3A and B, Table 3). Detection at the single-larva level was successful in all experiments regardless of extraction protocol (n = 5 for PowerSoil Kit, n = 4 for DNeasy Plant Kit). No *Carcinus*-specific amplification was observed in control extractions from unspiked ballast samples, despite successful DNA extraction (as indicated by successful universal COI amplification).

In a more demanding test of sensitivity, larvae were added to larger scale mock communities consisting of up to 1.359 grams (filtered weight) of mixed, cultured zooplankton. Even in these experiments, we were able to detect a single larva in over 1 gram of non-target biomass; detection was successful in all four experiments with 1, 5, 10, or 20 *C. maenas* larvae (Figure 3C and Table 3). The amount of final amplification product appeared to decrease with the number of target organisms in these experiments. For example, in the single larva experiment, the final product was considerably weaker than for the spiked ballast experiments.

Discussion

The European green crab *C. maenas*—and, to a lesser extent, it's congener *C. aestuarii*—has demonstrated its ability to successfully establish invasive populations that pose significant potential threats to recipient ecosystems. A number of vectors have been implicated in the anthropogenic translocation of both species beyond their native ranges (Cohen et al 1995; Carlton and Cohen 2003). Probably one of the most important contemporary vectors is the transport of larvae in ballast water. Given the duration of larval stages for *C. maenas*, it is likely that larvae could survive even lengthy transoceanic voyages (Cohen et al 1995; Carlton and Cohen 2003). Genetic evidence for the recent establishment of a *C. maenas* population in Nova Scotia suggests that the opening of new shipping lanes between northern Europe and the Strait of Canso Superport may have enabled the introduction of *C. maenas* to this region (Roman 2006); ballast water would thus be the most likely vector for this invasion. Similarly, ballast water discharge has been cited as the most probable source of invasive *C. maenas* populations in Argentina (Hidalgo et al 2005). Considering the impressive fecundity of *C. maenas* females (Broekhuysen 1936), entrainment, translocation, and discharge of larvae in ballast water could be a significant source of propagules for seeding introduced populations throughout the globe. Moreover, the natural dispersal of *C. maenas* larvae by offshore currents likely has contributed to the regional spread of the species within its introduced ranges. In Pacific North America, enhanced northward currents and warmer ocean temperatures accompanying periodic El Niño events almost certainly have facilitated the rapid expansion of the *C. maenas* population from San Francisco Bay to Vancouver Island (Yamada and Hunt 2000). A similar mechanism may have led to the expansion of *C. maenas* populations from southeastern Australia to Tasmania, although coastal shipping may provide an alternative explanation for this event (Thresher et al 2003).

It is important to note that the established introduced range of *C. maenas* is considerably smaller than the potential global range based on the environmental requirements of the species (Carlton and Cohen 2003). Continued transportation of *C. maenas* in ballast water and by other vectors thus has the potential to result in additional invasions across the globe. This fact, together with the potential importance of natural and anthropogenic larval dispersal to range expansion of established introduced populations, highlights the importance of detection and monitoring of *C. maenas* larvae in environmental samples as a tool for assessing and managing future risks associated with this species.

Utility of DNA-based methods for sensitive detection of targets

The important role of larval dispersal in the spread of marine invasive species has already prompted the development of several DNA-based tools for the rapid and sensitive detection of larvae and other propagules in environmental samples. This task necessitates the design of assays capable of discriminating target species from non-targets in a background that is potentially both diverse in biotic composition and overwhelming in terms of non-target biomass. The sensitivity of the PCR-based assay described here compares favorably with other assays reported in the literature for monitoring invasive species in environmental samples. Our ability to detect single first stage larvae in up to 178 mg of mixed plankton derived from ballast water (Figure 3) is comparable to the detection limits reported for other similar assays. Patil et al. (2005a) recently described the development of species-specific PCR assays for the detection of the toxic dinoflagellate *Gymnodinium catenatum* (Graham, 1943) in both ballast water and environmental plankton samples. Using this approach, as few as 5 *G. catanatum* cysts could be detected in approximately 131 mg of plankton (filtered weight), the equivalent of nearly 75 liters of filtered ballast water. Similar success

was achieved in developing PCR-based approaches for detecting larval forms of Pacific Oyster *Crassostrea gigas* (Thunberg, 1793) and the seastar *Asterias amurensis* (Lütken, 1871). In the case of *C. gigas*, specific detection of 5 D-hinge larvae or 50 earlier-stage larvae (ciliated blastulae) was possible in a background of approximately 150 mg mixed plankton (Patil et al 2005); for *A. amurensis*, detection limits were as low as one larva in 200 mg plankton (Deagle et al 2003). In another study, the availability of such tools allowed researchers to recognize the existence of a free-living larval form of a relatively poorly studied invasive gastropod species (Gunasekera et al 2005).

Given the facility with which we were able to detect single larvae in these samples, we pursued more demanding tests by spiking mock plankton samples containing several-fold higher levels of non-target biomass. These experiments demonstrate the ability of our assay to detect at extremely low levels, as low as a single larva in over 1 gram of mixed plankton (Figure 3). The sensitivity demonstrated in these experiments is significantly higher than published sensitivity estimates for other invasive species detection assays, likely reflecting both the specificity of PCR and the ease with which *Carcinus* DNA is recovered using standard, commercially available extraction methods. The composition of our mock plankton samples is unlikely to mimic any realistic environmental sample, being drawn from cultured stocks of both marine and freshwater zooplankton. However, the success of these experiments suggests that our assay is capable of specifically detecting *C. maenas* larvae in very large amounts of background biomass. The generation of weaker amplification products in these more demanding tests—a particularly weak band is observed when detecting a single larva in 1.184 grams of plankton (Figure 3)—indicates that we are likely approaching the detection limits of the assay.

Design of PCR-based detection assays

The development of the assay described here takes advantage of the considerable genetic information available for the target species. The ability to design highly species-specific DNA-based assays depends crucially on the amount of obtainable sequence data. Basing species-specific assay design on limited genetic data, though often necessary, raises the possibility of false negative results in the case of populations exhibiting unknown nucleotide variants not recognized by the assay. Previous genetic studies on *Carcinus* have generated abundant sequence data from the mitochondrial COI locus (Roman and Palumbi 2004), greatly facilitating assay development. Additional mtDNA sequencing (Darling, et al. unpublished data) provided us with a total of 99 *Carcinus* haplotypes from almost every known region within the genus' native and introduced ranges. The availability of such extensive sequence data is unusual for invasive species, and generates additional confidence in the utility of our assay for detecting *Carcinus* across the globe. In addition, the frequent adoption of COI as an informative locus for phylogenetic analysis and, more recently, DNA barcoding (Hebert et al 2003) increased the availability of multiple non-target DNA sequences necessary for development of the PCR assay. Sequence alignments indicate that our *Carcinus*-specific primers are unlikely to amplify from any of the non-*Carcinus* species investigated (Table 1); this is confirmed by direct testing of a number of non-target crab species (Figure 1 and Table 2). Importantly, many of the species tested exhibit ranges that overlap with that of the *Carcinus* species. In particular, we have tested many of the crab species likely to coexist with *C. maenas* along the Pacific coast of North America.

Our assay is capable of successfully discriminating between *C. maenas* and *C. aestuarii* in all tested cases (Figure 2), and sequence alignments suggest that the presence of the EcoNI site within the *Carcinus*-specific amplicon is truly diagnostic of *C. maenas* (Table 1). PCR-RFLP is an ideal approach for detecting multiple target species, and its utility has been repeatedly demonstrated. Weathersbee et al. (2003) recently adopted PCR-RFLP to distinguish between morphologically cryptic eggs of two closely related root weevils, the regulated invasive *Diaprepes abbreviatus* (Linnaeus, 1758) and the minor native pest *Pachnaeus litus* (Germar, 1824). In some cases, underlying variation has been sufficient even to target populations from specific geographic origins. Saltonstall et al. (2003), for instance, were able to develop a rapid and inexpensive means of distinguishing invasive and non-invasive haplotypes of the common reed *Phragmites australis* (Cav. (Trin.) ex Steud.) in North America. In another study, species-specific restriction sites and genus-specific PCR

primers allowed identification of both European and Asian varieties of introduced gypsy moths *Lymantria dispar* (Linnaeus, 1758) (Pfeifer et al. 1995). Our study thus contributes to a growing literature indicating the utility of the PCR-RFLP approach for the specific detection of invasive and pest species.

Given the number of different COI haplotypes that have been found within the genus, it was not possible to find any universally conserved regions large enough to design genus-specific PCR primers. This variation thus necessitated the design of primers that possess known nucleotide mismatches to certain target haplotypes. This problem was particularly pronounced for the reverse priming site. Every effort was made in the primer design process to limit these mismatches to the 5' end of the primer, while at the same time maintaining non-target nucleotide mismatches in the 3' end (see Table 1). This approach ensured that PCR amplification of all targets was possible even under the relatively stringent reaction conditions sufficient to prevent recognition of non-target template. Direct testing indicates that our assay successfully amplifies COI from *Carcinus* individuals possessing these mismatched haplotypes (Figure 1 and Table 2). This success demonstrates the possibility of developing specific PCR-based assays even when high levels of nucleotide variation preclude identification of universally conserved regions for primer design, a situation most likely to arise in the case of species for which considerable sequence information is available. Still, this difficulty underlines the possibility of this or any similar PCR-based assay encountering unrecognized haplotypes and generating false negative results. Due to the number and geographic range of available *C. maenas* haplotypes, it is likely that our dataset provides an excellent sampling of the existing genetic diversity for that species; the only unrepresented region was the species' putative Atlantic African range. In the case of *C. aestuarii*, it is more likely that additional mismatches may occur, as the native population is more poorly sampled.

Conclusions

Given the clear risks posed by *C. maenas* and the uncertainty surrounding the invasive capacity of *C. aestuarii*, detection and early monitoring of both species is clearly warranted for those regions at high risk. These include areas possessing environmental conditions conducive to green crab colonization and connected to already established populations either by current-driven dispersal (e.g. much of the Pacific coast of North America and South Australia), or by transoceanic shipping (e.g. Pacific South America and mainland east Asia) (Carlton and Cohen 2003; Cohen et al 1995). In many of these areas, such monitoring programs already exist. On the Pacific coast of North America, for example, management plans recommend biweekly or monthly sampling of uninvaded embayments (Grosholz and Ruiz 2002). Generally, however, such monitoring is limited to trapping postlarval juveniles, or "young of the year" crabs. The ability to detect the presence of larval crabs, either in the water column of uninvaded estuaries or in ballast water being released into those estuaries, should greatly improve forecasting and enable more direct assessment of the propagule pressure on at-risk ecosystems.

The general need for rapid, inexpensive, in situ monitoring tools for invasive species has prompted the development of DNA-based methods for specific and sensitive detection of target species in environmental samples. Tools such as the PCR-based assay described in this work represent only the first generation in the development of DNA-based technologies appropriate for invasive species management. They provide the foundation for exploration of more advanced approaches such as real-time PCR for quantification of target species abundance, microarray-based assays for detection of multiple targets in a single sample, or PCR-independent technologies appropriate for true "lab-on-a-chip" applications (Darling and Blum 2007). Even in their present form, however, assays such as that described here will enable early detection of potentially damaging invasions and monitoring of likely vectors and pathways of introduction, and will improve predictive models and risk assessments. In addition, by providing novel means of assessing larval transport such tools may prove valuable to researchers seeking to better understand the population dynamics of invasive species establishment and spread.

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